Fibroblast-specific integrin alpha V differentially regulates type 17 and type 2 driven inflammation and fibrosis

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Running Title: Fibroblast-specific integrin-alpha V regulates inflammation and fibrosis

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Abstract:

Fibroproliferative diseases affect a significant proportion of the world's population. Despite this, core mechanisms driving organ fibrosis of diverse etiologies remain ill defined. Recent studies suggest integrin-alpha V serves as a master driver of fibrosis in multiple organs. Although diverse mechanisms contribute to the progression of fibrosis, TGF-β and IL-13 have emerged as central mediators of fibrosis during type 1/type 17, and type 2 polarized inflammatory responses, respectively. To investigate if integrin-alpha V interactions or signaling is critical to the development of type 2 fibrosis, we analyzed fibroblast-specific integrin-alpha V knockout mice in three type 2-driven inflammatory disease models. While we confirmed a role for integrin-alpha V in type 17-associated fibrosis, integrin-alpha V was not critical to the development of type 2-driven fibrosis. Additionally, our studies support a novel mechanism through which fibroblasts, via integrin-alpha V expression, are capable of regulating immune polarization. We show that when integrin-alpha V is deleted on fibroblasts, initiation of type 17 inflammation is inhibited leading to a deregulation of type 2 inflammation. This mechanism is most evident in a model of severe asthma, which is characterized by a mixed type 2/type 17 inflammatory response. Together, these findings suggest dual targeting of integrin-alpha V and type 2 pathways may be needed to ameliorate fibrosis and prevent rebound of opposing pro-fibrotic and inflammatory mechanisms.

Key words:

Fibrosis, Inflammation, Asthma, Liver, Lung, Type 2, Type 17, Th2, Th17

Introduction:

Progressive tissue fibrosis is one of the leading causes of morbidity and mortality worldwide. Despite this, there are few effective approaches to treat fibroproliferative diseases, and currently, the only option for some end stage fibrotic diseases is organ transplantation. Transforming growth factor-β (TGF-β) is a potent family of cytokines with pleotropic effects, including the ability to influence T cell differentiation, suppresses inflammatory responses by acting on various leukocytes, and to induce fibrosis through the activation of collagen producing myofibroblasts (1). Direct targeting of TGF-β systemically is not generally viewed as therapeutically viable due to off target effects; therefore, the upstream and downstream mediators that control TGF-β activation and function have been studied intensively for their potential in anti-fibrotic therapies (2-4). Integrins are one such upstream target, with accumulating evidence suggesting they may serve as a therapeutic target to ameliorate collagen deposition in fibrotic diseases. During wound repair, integrins expressed by both resident cells and cells migrating towards chemoattractants released at the site of tissue injury can bind to and shear active TGF-β from the latency associated peptide through the contraction of actin-myosin stress fibers (5, 6). Integrins exist as cell surface heterodimers, composed of one alpha and one beta subunit that are able to bind to and interact with the extracellular matrix (7). Of the potential alpha subunits, only alpha V integrin (Itgav) has been shown to be critical for binding and activating TGF-β (5, 8). Variation within the beta subunit of the integrin dimer, by contrast, confers tissue specificity (9). Studies targeting Itgav on myofibroblasts have identified Itgav as a core molecular pathway regulating fibrosis in CCl_4-driven liver fibrosis, bleomycin-induced lung fibrosis, unilateral ureter obstruction-induced kidney fibrosis, and cardiac and skeletal muscle fibrosis, models largely driven by type 17 immune responses (10, 11). These studies identified integrin-mediated TGF-β activation as a promising target for fibroproliferative disease; hence, drugs targeting Itgav are in development.

We and others have previously shown that type 2 immunity, characterized by the effector cytokines IL-4, IL-5, IL-9, and IL-13, can induce fibrosis through TGF-β-dependent and independent mechanisms (12-14). Nevertheless, the role of integrins in the development of fibrosis during a more polarized type 2 cytokine driven inflammatory response is largely uncharacterized. Therefore, we tested the role of Itgav in several Th2-driven murine models of fibrosis including infection with the helminth *Schistosoma mansoni*, an *S. mansoni* egg-induced pulmonary granuloma model, and a model of severe asthma characterized by mixed type 2/type 17 inflammation (15). We found that in the presence of a strong type 2 fibrotic stimulus, deletion of Itgav had no effect on collagen deposition, suggesting the role of Itgav in fibrosis is limited to type 17-driven disease entities. Beyond the role of Itgav in fibrosis, we observed marked alterations in type 2 and type 17 inflammation, suggesting a novel mechanism through which stromal cells are capable of shaping the local inflammatory milieu. Specifically, we show that Itgav on fibroblasts is important for the induction of type 17 inflammation. When Itgav is depleted on fibroblasts, type 17 inflammation is significantly reduced, leading to a marked increase in type 2 inflammation. These findings have important therapeutic implications as they suggest Itgav targeting alone will not impact fibrosis associated with type 2 inflammation, and may in some conditions exacerbate these pathologies.

Results:

Fibroblast-specific Itgav deletion is not universally protective in models of liver fibrosis

To study the role of Itgav on fibroblasts in a type 2 inflammatory setting we utilized *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice previously characterized (11). Platelet derived growth factor receptor beta (Pdgfrb) is specifically expressed at high levels by both activated and quiescent fibroblasts, making *Pdgfrb*-cre a useful tool to target deletion of Itgav specifically to fibroblast populations. To investigate the role of Itgav in type 2-driven liver

fibrosis *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were infected with *S. mansoni* percutaneously via the tail with 35 cercariae (Figure 1A). Livers from these mice were harvested and analyzed at 12 weeks post infection following establishment of chronic disease with substantial fibrosis. Fibrosis was unchanged in *Pdgfrb-cre*$^{+}$ when compared to *Pdgfrb-cre*$^{-}$ as observed by gross picrosirius red staining (PSR), quantification of PSR staining, hydroxyproline content, as well as expression of collagen associated genes *Col3a1* and *Col6a1* (Figures 1B-E).

Previous studies used CCl_4-driven liver fibrosis, a type 17-/TGF-β-driven liver fibrosis model, to demonstrate the critical role of fibroblast-specific Itgav in the development of fibrosis (11, 16). To reconfirm the role of Itgav in type 17-driven liver disease *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were injected intraperitoneally with 1 µL per gram body weight CCl_4 twice weekly for 6 weeks (Figure 1F). We confirmed that *Pdgfrb-cre*$^{+}$ mice showed a dramatic reduction in fibrosis as observed through PSR staining, PSR quantification, hydroxyproline quantification, and *Col1a1 and Col3a1* expression (Figures 1G-J) when compared to *Pdgfrb-cre*$^{-}$ littermates receiving the same treatment. These data suggest that in the liver, type 2-driven fibrosis bypasses the pro-fibrotic mechanisms of Itgav and that this mechanism may be specific to type 17-driven disease insults.

Fibroblast-specific Itgav deletion is not protective in models of type 2-driven lung fibrosis

To determine the broader applicability of these results to other organ systems, we utilized an established secondary pulmonary granuloma model that produces a type 2-driven lung disease similar to that seen in the liver during *S. mansoni* infection. *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were sensitized intraperitoneally with 5,000 *S. mansoni* eggs and then challenged 14 days later intravenously with 5,000 live *S. mansoni eggs* by tail injection, which leads to deposition of eggs in the pulmonary capillary bed. Lungs were harvested 7 days post challenge at the peak of the inflammatory response (Figure 2A). Itgav deletion on fibroblasts resulted in no differences in

granulomatous lung fibrosis, as measured by PSR staining, hydroxyproline content, and *Col3a1* and *Col6a1* mRNA expression in the lung tissue (Figures 2B-D).

We next confirmed the importance of Itgav in type 17/ TGF-β -driven fibrosis in the lung using the bleomycin lung injury model (17). To do so, *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were administered 1.5 U/kg of bleomycin intranasally, and their lungs were harvested 28 days post challenge (Figure 2F). Fibrosis was significantly reduced in the *Pdgfrb-cre*$^{+}$ animals as observed by PSR staining and hydroxyproline content (Figures 2G, 2H). These results expand our findings in the liver (Figure 1) to the lung, showing that Itgav is critical to type 17-driven fibrosis but is bypassed in type 2-driven disease models.

Itgav deletion on fibroblasts causes alterations in immune polarization in both the lung and liver

Fibroblast-specific Itgav has previously been shown to drive fibrosis through TGF-β activation. In addition to its role in fibrosis, TGF-β is a potent immunoregulatory cytokine and an important differentiation factor for Th17 cells. Therefore, immunophenotypic analysis was performed to determine if fibroblasts, via expression of Itgav, regulate the local inflammatory milieu. *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were infected *with S. mansoni* and immune responses were characterized 12 weeks post-infection in the liver (Figure 3A). Liver leukocytes were collected and re-stimulated ex-vivo for analysis by intracellular cytokine staining and flow cytometry. The frequency of IL-17A and IL-13 producing CD4^{+} T cells were not significantly altered in *Pdgfrb-cre*$^{+}$ compared *Pdgfrb-cre*$^{-}$ mice (Figure 3B). We also assessed cellular determinants associated with type 2 and type 17 inflammation, namely tissue eosinophil and neutrophil accumulation, respectively. We did not detect any significant differences in liver neutrophils between the *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ animals. However, in mice lacking Itgav on fibroblasts, eosinophil accumulation was significantly increased (Figure 3B).

We also performed immune characterization in lungs from animals subjected to the secondary pulmonary granuloma model (Figure 3C). The frequency of IL-13 producing $CD4^+$ T cells was not significantly altered in the *Pdgfrb-cre*$^+$ compared to *Pdgfrb-cre*$^-$ mice. However, the frequency of IL-17A-producing $CD4^+$ T cells was significantly reduced in the *Pdgfrb-cre*$^+$ compared to *Pdgfrb-cre*$^-$ animals (Figure 3D). While we did not detect significant differences in lung neutrophils between the *Pdgfrb-cre*$^+$ and *Pdgfrb-cre*$^-$ animals, overall frequencies were decreased relative to naïve animals due to the overwhelming induction of type 2 inflammation in this response (of $CD4^+$ population: 23.54% T_H2 vs 0.81% T_H17). However, in mice lacking Itgav on fibroblasts, eosinophil accumulation was again significantly increased (Figure 3D). Together, these findings (Figure 3A-D) suggest fibroblast-specific Itgav alters local inflammation in type 2-driven liver and lung disease.

To expand these findings to type 17-driven models, the CCl_4 liver fibrosis model was utilized (Figure 3E). Analysis of the inflammatory readouts in this model demonstrated a reduction in IL-17A-producing $CD4^+$ T cells in *Pdgfrb-cre*$^+$ animals, and a concomitant increase in IL-13-producing $CD4^+$ T cells. We did not observe changes in neutrophil or eosinophil cell frequencies (Figure 3F).

To investigate if Itgav on fibroblasts influences inflammatory character in type 17-driven lung disease, the bleomycin-induced lung fibrosis model was used (Figure 3G). No significant differences in the frequency of IL-17A-producing $CD4^+$ T cells from re-stimulated lung leukocytes were observed. However, the frequency of IL-13 producing $CD4^+$ T cells increased significantly in *Pdgfrb-cre*$^+$ animals. Nevertheless, analysis of infiltrating cell populations revealed a reduction in both $Ly6G^+$ neutrophils and Siglec-F^+ eosinophils in the bronchoalveolar lavage (Figure 4H).

These data suggest that while presence of Itgav on fibroblasts may not universally impact the development of fibrosis, it can regulate the balance between type 2 and type 17 inflammation, which we and others have demonstrated can play a critical role in the lung (18). Additionally, we determined that the ability of Itgav to alter inflammation is not limited to type 17 models, as similar inflammatory changes are seen in both type 17 and type 2 disease models. Nevertheless, given that the bleomycin and CCl_4 models of fibrosis do not depend on antigen driven T cell activation and associated cytokine responses, as seen in other models used here including the helminth infections, our ability to detect consistent shifts in type-1/type-2 inflammation with these models may be more difficult to discern.

Itgav deletion on fibroblasts inhibits type 17 inflammation and induces a compensatory type 2 response in cGMP/HDM asthma model

To this end, we took advantage of a model of severe asthma driven by intranasal instillation of low dose house dust mite extract (HDM) combined with the STING ligand cyclic-di-GMP (cGMP). Unlike the *S. mansoni*, bleomycin, and CCl_4 models, this model has high levels of type 2/type 17 mixed inflammation, making it ideal to investigate the inflammatory changes observed when Itgav is deleted on fibroblasts (15). *Pdgfrb-cre*$^+$ and *Pdgfrb-cre*$^-$ littermates were sensitized with HDM and cGMP and subsequently challenged with HDM and a lower dose of cGMP (Figure 4A). While we did not detect any differences in fibrosis measured by hydroxyproline between *Pdgfrb-cre*$^+$ and *Pdgfrb-cre*$^-$ animals (Figure 4B), the type 17 response was significantly decreased in *Pdgfrb-cre*$^+$ animals as assessed by the frequency of IL-17A-producing $CD4^+$ T cells in the lung as well as total tissue IL-17A expression (Figures 4C, 4D). To determine if this corresponded with a functional decrease in the IL-17A pathway, we assessed lung expression of the IL-17A responsive neutrophil chemoattractant genes *Cxcl1* and *Cxcl5,* and observed significant reductions in both (Figure 4E).

Additional analysis of infiltrating tissue and BAL cell populations demonstrated significantly fewer tissue and BAL neutrophils in *Pdgfrb-cre*$^{+}$ animals in agreement with the decreased expression of the neutrophil chemoattractants (Figure 4F).

We next asked if the impaired initiation of type 17 inflammation observed in animals with Itgav deletion on fibroblasts was accompanied by a dysregulation and increased presence of type 2 inflammation as we have previously observed in a chronic HDM model of allergic asthma when mice were treated with a neutralizing mAb to IL-17A (18). Indeed, compared with *Pdgfrb-cre*$^{-}$ animals, *Pdgfrb-cre*$^{+}$ mice exhibited an increase in type 2 inflammatory markers as measured by significant increases in IL-13^{+} CD4^{+} T cells isolated from lung tissue of the cGMP/HDM mice as well as total tissue *Il13* expression (Figures 4G, 4H). The increased IL-13 response also correlated with increased eosinophil accumulation in both lung tissue and BAL (Figure 4I), increased expression of the eosinophil chemoattractant *Ccl11* and the type 2 immune marker *Chi3l3*. Additionally, IL-13-induced mucus-associated genes, *Muc5ac* and *Gob5* were increased in the lung tissue of *Pdgfrb-cre*$^{+}$ animals (19-21) (Figure 4J). Mucus production was also increased, as assessed by AB/PAS tissue staining (Figure 4K). Additionally, through flexiVent analysis, we observed a significant reduction in lung resistance when Itgav was deleted on fibroblasts, suggesting improved airway hyperreactivity (AHR) (supplemental figure 1A). These data indicate that fibroblast specific deletion of Itgav dysregulates the inflammatory environment, favoring type 2 inflammation, reducing airway resistance but ultimately resulting in worsening of disease-associated eosinophilia and mucus production. The observed reduction in the type 17-signature accompanied by increased type 2-inflammation and mucus production reveals a novel, unexpected, yet pleotropic role for fibroblasts in regulating inflammation, immune balance, and measures of clinically relevant disease severity.

Itgav deletion on fibroblasts in conjunction with IL-13 neutralization inhibits Th17 inflammation while inhibiting a compensatory Th2 response in cGMP/HDM asthma model

In order to better understand the mechanism underlying fibroblast processing of TGF-β through Itgav and its regulation of the immune response, we asked how *Pdgfrb-cre*$^{+}$ animals would behave if the rebound type 2 inflammation was blocked using an IL-13-specific antibody in the same cGMP/HDM model of severe asthma. *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ littermates were treated with either anti-IL-13 or an IgG isotype control twice weekly (Figure 5A). No changes in fibrosis between the *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ animals were observed, and anti-IL-13 treatment did not significantly affect fibrosis as shown by hydroxyproline quantification (Figure 5B). We observed the same trend of decreased type 17 inflammation in *Pdgfrb-cre*$^{+}$ animals as seen in previous experiments (Figure 4), with anti-IL-13 antibody treatment having minimal effect on the type 17 signature as assessed by total tissue *Il17a* expression, frequency of IL-17A-producing CD4^{+} T-cells in the lung, total tissue and BAL neutrophil quantification, and expression of neutrophil chemoattractants *Cxcl1* and *Cxcl5* (Figures 5C-F).

We next assessed what effect anti-IL-13 treatment would have on the rebound type 2 inflammatory response seen in *Pdgfrb-cre*$^{+}$ animals in the previous experiments (Figure 4). Again, we observed that the type 2-signature was significantly increased in the absence of Itgav. However, IL-13 antibody treatment ablated this compensatory increase in *Pdgfrb-cre*$^{+}$ mice as measured by total tissue *Il13* expression, frequencies of IL-13^{+} CD4^{+} T-cells, total lung tissue and BAL eosinophil frequency, the eosinophil chemoattractant *Ccl11*, and the mucus-associated gene *Muc5ac* (Figures 5G-I). The increased mucus production in *Pdgfrb-cre*$^{+}$ animals receiving cGMP/HDM treatment was also reduced in anti-IL-13 treated animals (Figure 5J). Together, these data reveal an important role for fibroblast-specific Itgav expression in the coordination and regulation of type 17 and type 2–driven inflammatory responses.

Discussion:

Recent publications have shown Itgav to be part of a fundamental pathway regulating fibrosis in the liver, lung, skin, kidney, and cardiac and skeletal muscle (9). Blockade of either Itgav or one of its five beta subunits, via genetic deletion or chemical blockade, substantially attenuated fibrosis (9, 11). In these studies, Itgav blockade was efficacious both prophylactically and therapeutically. However, these previous studies did not explore the importance of Itgav in settings where type 2 cytokines are believed to function as important drivers of disease progression. In this study, we compared mice with or without Itgav on fibroblasts and observed that type 2 fibrosis driven by *S. mansoni* infection or egg-induced granuloma formation is independent of fibroblast-specific Itgav expression in the liver and the lung, respectively. In these type 2-driven disease models, integrin activation of TGF-β by fibroblasts played little to no detectable role in fibrosis. Nevertheless, we confirmed that fibrosis driven predominantly by type-17/TGF-β, including bleomycin- and CCl_4-driven fibrosis, were highly Itgav dependent (17).

Unexpectedly, however, we discovered that Itgav deficiency on fibroblasts resulted in a substantial decrease in type 17 inflammation and corresponding increase in type 2 inflammation, particularly when a mixed type 17/type 2 inflammatory response was observed. The shift from type 17/neutrophilic to type 2/eosinophilic inflammation was most striking in the cGMP/HDM model of severe asthma, which was characterized as mixed type 17/type 2 inflammatory reaction (15). Similarly, a recent publication by Choy et al. showed that Th2 and Th17 inflammatory pathways are reciprocally regulated in both murine and human asthmatic lungs characterized by infiltration of their associated cellular determinants eosinophils and neutrophils, respectively. The results presented here suggest that the balance of these inflammatory pathways can be regulated, in part, by fibroblast-specific Itgav expression. Robust regulation of type-17 inflammatory readouts

mediated by Itgav or Itgb8 expression on dendritic cells has been described previously in EAE as well as several asthma models, and was associated with significantly reduced AHR (22-24). However, there were no reported changes in mucus or neutrophil and eosinophil counts despite a near complete inhibition of Th17 development. Surprisingly, despite less dramatic reductions in Th17 differentiation, fibroblast deletion of Itgav resulted in similar reductions in AHR as the Itgb8 deletion on dendritic cells that was previously reported. Similarly, global blockade of avb8 with a neutralizing antibody was shown to inhibit IL-17, BAL neutrophils and AHR in a smoke inhalation model; although, it is unclear whether these effects were mediated via dendritic cells, fibroblasts, or both (25). Additional studies by Kitamura et al. demonstrated fibroblast deletion of Itgb8 resulted in decreased lung neutrophils and Th17 differentiation in response to adenoviral IL-1b and reduced inflammation and mucus in an ovalbumin challenge lung model that was not associated with decreased IL-17 or changes in neutrophils (26). AHR was not measured in those experiments and it is unclear if the additional modulation of the type-2 response, downstream cellular determinants and mucus we report are specific to the disease model or are mediated thru an alternate beta integrin. Taken together these data suggest some potential similarities in mechanisms regulating type-17 inflammation employed by dendritic cells and fibroblasts via integrins, but they are also suggestive of important differences in cell specific roles that may depend on Itgav pairing partners and disease context.

This immune regulation by fibroblasts via Itgav appears to be similar as was recently described for dendritic cells (23, 24). Our data demonstrate a role for fibroblasts regulating not only the intensity but also the balance of inflammatory pathways through integrin expression, expanding on a previous study invoking an Itgb8-mediated role for lung fibroblasts in the induction of inflammation in response to IL-1β (26). The inflammatory changes detected in *Pdgfrb-cre*$^{+}$ mice may be the result of reduced Th17 induction and a compensatory induction and deregulation of

the type 2 response. Furthermore, TGF-β is known to support the differentiation of Th0 cells into Th17 cells and regulatory T cells (27-29). Therefore, the reduction of a major source of active TGF-β through Itgav deletion on fibroblasts may impair Th17 differentiation and license these cells towards other lineages. These findings posit a novel role for fibroblasts in the immune polarization decision-making process through Itgav expression.

These findings also have important therapeutic implications as they suggest targeting Itgav may inadvertently skew inflammatory responses towards type 2 inflammation and alternative pro-fibrotic mechanisms, particularly when stimuli promoting these alternative pathways are also present. Previous studies by our group demonstrated that dual blockade of IL-13 and IL-17A in preclinical asthma models is more efficacious because it limits the rebound inflammation that occurs during mono-therapy. Similarly, in the cGMP/HDM model used in this current study, increased type 2 responses in the absence of fibroblast-specific Itgav led to increased eosinophilia and mucus production, key functional contributors to severe asthmatic disease (30-34). Our flexiVent data demonstrate that targeting Itgav on fibroblasts decreases airway resistance in the cGMP/HDM severe asthma model. This agrees with some reports that neutrophils, not eosinophils, may be the critical drivers of AHR (35-39). Nevertheless, despite the improvements in AHR, concomitant upregulation of type 2 immune responses resulted in significant eosinophilia and mucus production. Mucus hypersecretion is a hallmark of chronic airway diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Studies examining the effect of mucus on lung pathology show that mucus overproduction is directly correlated to progressive declines in lung function, worsened quality of life, hospitalizations, and mortality (40-56). Therefore, we believe that while AHR may be decreased, the induction of mucus overproduction is of clinical importance and should be considered during treatment and clinical trial development. Overall, these results indicate that while fibroblast-specific expression of Itgav is a critical driver of

Th17 inflammation in the asthmatic lung, therapeutic targeting of Itgav may require a coordinated treatment strategy to prevent compensatory rebound type 2 inflammation. Increasing evidence suggests that the emergent type 17 inflammation in treatment-resistant severe asthma may be associated with potent corticosteroid control of the type 2 response (18, 57, 58). The effectiveness of Itgav deletion on fibroblasts to control Th17 induction indicates it may serve as an ideal paired therapy with standard therapy or novel blockers of type 2 inflammation. Indeed, in our studies, administration of anti-IL-13 to animals lacking Itgav on fibroblasts reduced eosinophilia and mucus production to levels of saline controls. These data suggest that dual blockade of Itgav and IL-13, or other mediators of type 2 immunity may represent a more efficacious therapeutic strategy for treatment-resistant asthmatics.

We conclude that in fibrotic diseases of diverse etiologies, the role of fibroblast-specific Itgav in fibrosis is highly dependent on the inflammatory stimuli driving the response. In addition, these data reveal a new role for Itgav expression on stromal cells, as they show it plays a critical role in shaping the character of local inflammatory responses. Thus, while targeting Itgav in fibrotic disease may be efficacious in some settings, it will be important to understand the inflammatory mechanisms driving the fibrogenic response in order to better predict clinical outcome. Our findings suggest Itgav inhibition may be utilized to attenuate type 17 inflammation in diseases such as severe asthma. While type-17 inflammatory pathways may be downregulated by Itgav blockade, the potential for an undesirable augmentation of type-2 inflammation and resulting pathology should be monitored carefully. Indeed, in settings where there is high risk of compensatory type 2 inflammation, dual blockade strategies may be required to maximize therapeutic benefit.

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Materials and Methods:

Mice

Pdgfrb$^{cre/wt}$*Itgav*$^{flox/flox}$ were obtained from Neil Henderson (11); animals used in studies were age and gender matched, littermates and between 6-8 weeks of age. Mice were terminally anesthetized with sodium pentobarbital. All animals were housed under specific pathogen-free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care-approved facility.

Schistosoma mansoni Infection

Mice were infected percutaneously by suspending tails in pond water containing 35 *S. mansoni* cercariae for 45 minutes. Cercariae were obtained by shedding infected *Biomphalaria glabrata* snails (Biomedical Research Institute).

Pulmonary Granuloma Model

S. mansoni eggs were extracted from the livers of infected mice (Biomedical Research Institute, Rockville, MD, USA). For the induction of secondary lung granulomas, mice were sensitized intraperitoneally (i.p.) with 5,000 *S. mansoni* eggs and then challenged 14 days later with 5,000 live *S. mansoni* eggs i.v.

370

371 **CCl_4 and Bleomycin Fibrosis Models**

372 For chronic CCl_4–induced liver fibrosis, mice were injected i.p. with 1 µl CCl_4 (Sigma)/gram body
373 weight or olive oil twice a week for 6 weeks. To induce pulmonary fibrosis, mice were
374 anesthetized, and saline or with 1.5 U bleomycin (Sigma)/kg body weight was instilled
375 intratracheally.

376

377 **cGMP/HDM Model of Severe Asthma**

378 Mice were sensitized to 25 µg HDM (greer labs) extract and 5 µg cyclic-di-GMP (cayman chemical
379 company) delivered intranasally on days 1, 3, and 5. Mice were rested 5 days, then intranasally
380 administered with 3 challenge sets consisting of 3 consecutive daily challenges with HDM and
381 cyclic-di-GMP with 4 days of rest between each set. Each challenge set included 0.5 µg cyclic-di-
382 GMP with 25 µg HDM on day 1, then 25 µg HDM on the following 2 days.

383

384 **Histopathology**

385 Murine liver and lung lobes were harvested and fixed with Bouin's-Hollande solution. Fixed tissue
386 was embedded in paraffin for sectioning, and stained (Histopath of America, Clinton, MD) with
387 Wright's Giemsa, Picrosirius red (PSR), or Alcian Blue Periodic Acid Schiff (AB/PAS).

388

389 **Fibrosis Quantification**

390 Cross sections of three liver lobes from each mouse were stained with PSR and imaged under
391 polarized light. Granuloma volume was obtained by measuring the length (a) and width (b) of
392 granulomas with a single visible egg. Values obtained were entered into the formula of a spheroid
393 $(\frac{4}{3}\pi ab^2)$.

394

IL-13 Neutralization

For antibody therapy, 250 μg of anti–IL-13 (Genentech clone 262A-5-1), or control Ab (BioXCell clone MOPC-21, catalog #BE0083) was injected intraperitoneally twice weekly.

Hydroxyproline Assay

Mouse liver tissue (200 mg) or the left lower lung lobe incubated overnight at 110 °C in 6M HCl. Hydroxyproline content was measured using a colorimetric chloramine T assay (59).

Cell Isolation and Flow Cytometry

Approximately 400 mg of liver or lung tissue was diced and incubated in 100 U/ml of collagenase (Sigma) at 37°C for an hour with rocking. Tissue was then passed through a 70-μm nylon filter to obtain a single cell suspension. Leukocytes were isolated on a 40% Percoll (Sigma) gradient, and treated with ACK lysis buffer to remove erythrocytes. Isolated cells were either immediately stained for cellular analysis or stimulated with phorbol 12-myristate 13-acetate (10ng/ml) and ionomycin (1 μg/ml) in the presence of Brefeldin A (10 μg/ml) for three hours and fixed. Cells were permeabilized (Cytofix/Cytoperm buffer; BD Biosciences; San Diego, CA) and stained for 30 minutes with fluorescently labelled antibodies purchased from eBioscience (Waltham, MA) include the following: CD45(30-F11; 1:250) and IL-13(eBio13A; 1:150). Antibodies purchased from Biolegend (San Diego, CA) include the following: CD16/CD32 (93; 1:500), CD11b (M1/70; 1:250), and IL-17A(TC11-18H10.1; 1:150). Antibodies purchased from BD Pharmingen (Billerica, MA) include the following: CD4 (RM4-5; 1:150), Ly6C (AL-21; 1:250), ly6G(1A8; 1:350), and Siglec-F (E50-2440; 1:275). Cells were collected on an LSR II flow cytometer equipped with FACSDIVA (BD Biosciences) software and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Murine Gene Expression Analyses

Liver and lung tissue was homogenized in TRIzol Reagent (Life Technologies; Grand Island, NY) with a Precellys 24 (Bertin Technologies; Montigny-le-Bretonneux, France). Total RNA was extracted with chloroform using a MagMax-96 Total RNA Isolation Kit (Qiagen), and reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Life Technologies). Gene expression was quantified using Power SYBR Green PCR Master Mix (Applied Biosystems) by RT-PCR on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Gene expression is described relative to RPLP2 mRNA levels in naïve liver and lung tissue.

Gene	Forward Sequence 5' - 3'	Reverse sequence 5'-3'
Ccl11	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
Chil3	CATGAGCAAGACTTGCGTGAC	GGTCCAAACTTCCATCCTCCA
Col3a1	AACCTGGTTTCTTCTCACCCTTC	ACTCATAGGACTGACCAAGGTGG
Col6a1	CGCCCTTCCCACTGACAA	GCGTTCCCTTTAAGACAGTTGAG
Cxcl1	CTGGGATTCACCTCAAGAAC	GAAGCCAGCGTTCACCAGAC
Cxcl5	TGCGTTGTGTTTGCTTAACCG	AGCTATGACTTCCACCGTAGG
Gob5	AGGAAAACCCCAAGCAGTG	GCACCGACGAACTTGATTTT
Il13	CCTCTGACCCTTAAGGAGCTTAT	CGTTGCACAGGGGAGTCTT
Il17a	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
Muc5ac	CAGGACTCTCTGAAATCGTACCA	AAGGCTCGTACCACAGGGA

Statistical Analysis

Experimental groups were randomized and researchers were blinded to the groups. Prism 7 was used to compute statistical analyses. Data was tested for normal distribution and two-tailed Welch's t test or one-way ANOVA were used to determine statistical significance. A p value <0.05 was deemed statistically significant**.**

Ethics

The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all of the experimental procedures (protocol "LPD 16E"). The program complies with all applicable provisions of the Animal Welfare Act

439 (www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf) and other federal statutes and

440 regulations relating to animals.

Figure 1. Fibroblast-specific Itgav deletion is not universally protective in models of liver fibrosis

(A) *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were infected with *Schistosoma Mansoni* percutaneously via the tail with 25–35 cercariae. Livers were harvested 12 weeks post infection. (B) 5-µm sections of paraffin-embedded liver tissue were stained with PSR. (C) Quantification of positive PSR staining, expressed as percentage of pixels positive for stain. From left to right: n = 4, 4, 9, 6. (D) Liver collagen deposition, expressed as micromoles of hydroxyproline per gram of liver. From left to right: n = 4, 4, 9, 6. (E) RNA was extracted from liver tissue, with *Col3a1*, and *Col6a1* quantified by quantitative RT-PCR. From left to right: n = 4, 4, 8, 6. (F) *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ mice were injected i.p. with 1µl per gram body weight CCl_4 twice weekly for 6 weeks. (G) 5-µm sections of paraffin-embedded liver tissue were stained with PSR. (H) Quantification of positive PSR staining, expressed as percentage of pixels positive for stain. From left to right: n = 5, 9, 10. (I) Liver collagen deposition, expressed as micromoles of hydroxyproline per liver. From left to right: n = 4, 5, 14, 15. (J) RNA was extracted from lung tissue, with *Col1a1* and *Col3a1*mRNA quantified by quantitative RT-PCR. From left to right: n = 3, 3, 9, 10. Data representative of two replicate experiments; all scale bars represent 100 µm; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 2. Fibroblast-specific Itgav deletion is not protective in models of type 2-driven lung fibrosis

(A) *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ mice were sensitized intraperitoneally with 5,000 S. mansoni eggs and then challenged 14 days later intravenously with 5,000 live S. mansoni eggs. Lungs were harvested 7 days post challenge. (B) 5-µm sections of paraffin-embedded lung tissue were stained with PSR. (C) Pulmonary collagen deposition, expressed as micromoles of hydroxyproline per lung. From left to right: n = 4, 4, 9, 15. (D) Quantification of granuloma volume. From left to right: n = 4, 4, 4, 4. (E) RNA was extracted from lung tissue, with *Col3a1*, and *Col6a1* quantified by quantitative RT-PCR. From left to right: n = 3, 3, 4, 6. (F) *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were administered 1.5U bleomycin i.n. and lungs were harvested 28 days later. (G) 5-µm sections of paraffin-embedded lung tissue were stained with picrosirius red. (H) Pulmonary collagen deposition, expressed as micromoles of hydroxyproline per lung. From left to right: n = 6, 6, 12, 9.

Data representative of two replicate experiments; all scale bars represent 100 µm; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 3. Itgav deletion on fibroblasts causes alterations in immune polarization in both the lung and liver

(A) *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were infected with *Schistosoma Mansoni* percutaneously via the tail with 25–35 cercariae. Livers were harvested 12 weeks post infection (B) Liver lymphocytes were collected from *S. Mansoni* infected animals and re-stimulated ex-vivo for analysis by intracellular staining for IL-13, IL-17A, Ly6G, and Siglec-F and analyzed via flow cytometry. From left to right: n = 4, 4, 8, 6. (C) *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ mice were sensitized intraperitoneally with 5,000 S. mansoni eggs and then challenged 14 days later intravenously with 5,000 live *S. mansoni* eggs. Lungs were harvested 7 days post challenge. (D) Lung lymphocytes were collected from mice, subjected to secondary lung granuloma, model and re-stimulated ex-vivo for analysis by intracellular staining for IL-13, IL-17A, Ly6G, and Siglec-F and analyzed via flow cytometry. From left to right: n = 5, 4, 9, 14. (E) *Pdgfrb-cre*$^{+}$ and *Pdgfrb-cre*$^{-}$ mice were injected i.p. with 1µl per gram body weight CCl_4 twice weekly for 6 weeks. (F) Liver lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for IL-17A, IL-13, Ly6G, and Siglec-F and analyzed via flow cytometry. From left to right: n = 4, 5, 4. (G) *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were administered 1.5U bleomycin i.n. and lungs were harvested 28 days later. (H) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for IL-17A, IL-13, Ly6G, and Siglec-F and analyzed via flow cytometry. From left to right: n = 3, 5, 14, 10.

Data representative of two replicate experiments; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure 4. Itgav deletion on fibroblasts inhibits Th17 inflammation and induces a compensatory Th2 response in cGMP/HDM asthma model

(A) *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were sensitized with house dust mite allergen and ci-di-GMP and subsequently challenged with HDM and a lower dose of ci-di-GMP. (B) Pulmonary collagen deposition, expressed as micromoles of hydroxyproline per lung. From left to right: n = 6, 7, 12, 13. (C) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for IL-17A. From left to right: n = 5, 6, 12, 12. (D-E) RNA was extracted from lung tissue, with *Il17a*, *Cxcl1* and *Cxcl5* mRNA quantified by quantitative RT-PCR. From left to right: n = 5, 6, 11, 12. (F-G) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for Ly6G, and IL-13 and analyzed via flow cytometry. From left to right: n = 6, 7, 10, 12. (H) RNA was extracted from lung tissue, with *Il13* mRNA quantified by quantitative RT-PCR. From left to right: n = 4, 4, 5, 5. (I) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for Siglec-F and analyzed via flow cytometry. From left to right: n = 6, 7, 10, 12. (J) RNA was extracted from lung tissue, with *Ccl11*, *Chil3, Muc5ac* and *Gob5* mRNA quantified by quantitative RT-PCR. From left to right: n = 5, 6, 11, 12. (K) 5-µm sections of paraffin-embedded lung tissue were stained with AB/ PAS. Data representative of two replicate experiments; all scale bars represent 100 µm; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 5 Itgav deletion on fibroblasts in conjunction with IL-13-neutralization inhibits Th17 inflammation while inhibiting a compensatory Th2 response in cGMP/HDM asthma model

(A *Pdgfrb*$^{cre/wt}$*Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{+}$) and *Itgav*$^{flox/flox}$(*Pdgfrb-cre*$^{-}$) mice were sensitized with house dust mite allergen and ci-di-GMP and subsequently challenged with HDM and a lower dose of ci-di-GMP. 250ug of anti-IL-13 was also administered twice weekly. (B) Pulmonary collagen deposition, expressed as micromoles of hydroxyproline per lung. From left to right: n = 6, 6, 11, 11, 14, 13. (C) RNA was extracted from lung tissue, with *Il17a* mRNA quantified by quantitative RT-PCR. From left to right: n = 6, 4, 8, 8, 14, 12. (D-E) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for IL-17A and Ly6G. From left to right: n = 5, 3, 10, 9, 14, 12. (F-G) RNA was extracted from lung tissue, with *Cxcl1, Cxcl5,* and *Il13* mRNA quantified by quantitative RT-PCR. From left to right: n = 6, 4, 8, 8, 14, 12. (H) Lung lymphocytes were collected and re-stimulated ex-vivo for analysis by intracellular staining for IL-13 and Siglec-F and analyzed via flow cytometry. From left to right: n = 6, 4, 8, 8, 14, 12. (I) RNA was extracted from lung tissue, with Ccl11 and *Muc5ac* mRNA quantified by quantitative RT-PCR. From left to right: n = 6, 4, 8, 8, 14, 12. (J) 5-µm sections of paraffin-embedded lung tissue were stained with AB/PAS. Data representative of two replicate experiments; all scale bars represent 100 µm; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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